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How the Redox State Regulates Immunity

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Abstract

Oxidative stress is defined as an imbalance between the levels of reactive oxygen species (ROS) and antioxidant defences. The view of oxidative stress as a cause of cell damage has evolved over the past few decades to a much more nuanced view of the role of oxidative changes in cell physiology. This is no more evident than in the field of immunity, where oxidative changes are now known to regulate many aspects of the immune response, and inflammatory pathways in particular. Our understanding of redox regulation of immunity now encompasses not only increases in reactive oxygen and nitrogen species, but also changes in the activities of oxidoreductase enzymes. These enzymes are important regulators of immune pathways both via changes in their redox activity, but also via other more recently identified cytokine-like functions. The emerging picture of redox regulation of immune pathways is one of increasing complexity and while therapeutic targeting of the redox environment to treat inflammatory disease is a possibility, any such strategy is likely to be more nuanced than simply inhibiting ROS production.

1. Introduction

Inflammatory disease is often associated with oxidative changes. Some of the earliest reports in terms of the biochemical mechanisms involved were the identification of arachidonic acid oxidation products [1] and the inhibitory effects of antioxidants on the production of prostaglandins and thromboxanes [2]. These findings were based on even

earlier studies on rancidification of lipids in food and, were similarly measured by following the formation of malondialdehyde (MDA), also defined as thiobarbituric acid-reactive substance [3, 4].

A second aspect is that reactive oxygen species (ROS) produced by neutrophils during the oxidative burst to kill bacteria [5] can cause collateral damage and inflammation [6]. Pioneering studies by McCord have also implicated ROS in postischemic tissue injury [7], thus broadening the list of diseases for which oxidative stress is implicated. This prompted the hypothesis that low-molecular weight antioxidants [8], as well as antioxidant enzymes [9], could ameliorate inflammatory and immune-mediated disease.

With the discovery of inflammatory cytokines in the 1980s [10, 11] and the success of the cytokine theory of disease with the approval of anti-TNF antibodies for the treatment of rheumatoid arthritis and Crohn's disease, it was only natural that researchers investigated the effect of antioxidants and oxidants on cytokine production. We reported that endogenous glutathione (GSH) is an inhibitor of TNF production [12], a study that was followed by others showing inhibitory effects of antioxidants on inflammatory cytokine production [13].

However, a molecular mechanism for this effect was clarified only in the early 1990s in the laboratory of Patrick Baeuerle, with the finding that antioxidants inhibit, and ROS activate, the transcription factor NF- κ B, which is involved in the transcription of many inflammatory genes, including cytokines [14, 15].

2. From the concept of oxidative stress to those of redox regulation and eustress

NF- κ B was first described in 1986 as a transcription factor acting on immunoglobulin genes in B cells [16]. The first connection with inflammation was the discovery that TNF activates NF- κ B [17]. Later, it was found that NF- κ B activates the transcription of TNF [18] and other cytokines [19] in response to inflammatory stimuli. NF- κ B entered the redox arena in 1991 with a paper from Baeuerle's lab which showed for the first time that NF- κ B is activated by H₂O₂ and its activation by inflammatory stimuli is inhibited by thiol containing compounds including N-acetylcysteine [14]. This paper has since been cited over 4100 times. This study

raised the important point that apparently ROS can function exclusively as second messengers only in a narrow range of concentrations, while at higher concentrations are mainly toxic [14].

3. Molecular mechanisms of redox regulation: the role of protein thiols

The seminal study by Schreck et al. [14] describing the effects of ROS on activation of NF- κ B did not attempt to investigate the mechanism by which NF- κ B is redox regulated; the mechanism by which NF- κ B is redox regulated was simply depicted by an arrow with no further detail given. We will focus here on oxidoreduction of cysteine residues in proteins, in particular to form intramolecular disulfides or mixed disulfides with glutathione (protein glutathionylation).

In fact, while the classical explanation found in textbooks is that protein cysteines can exist in two forms, either as free thiols or engaged in a structural disulfide to hold together the 3D structure of the protein, there are many more forms of cysteine oxidation. Those that are reversible are clear candidates for redox regulatory mechanisms. The essence of this aspect of redox regulation is that these post-translational modifications can regulate protein activity. Figure 1 outlines two examples of cysteine oxidation: formation of intra-protein disulfide and glutathionylation, together with the similarities to the well-known regulatory mechanism of protein phosphorylation.

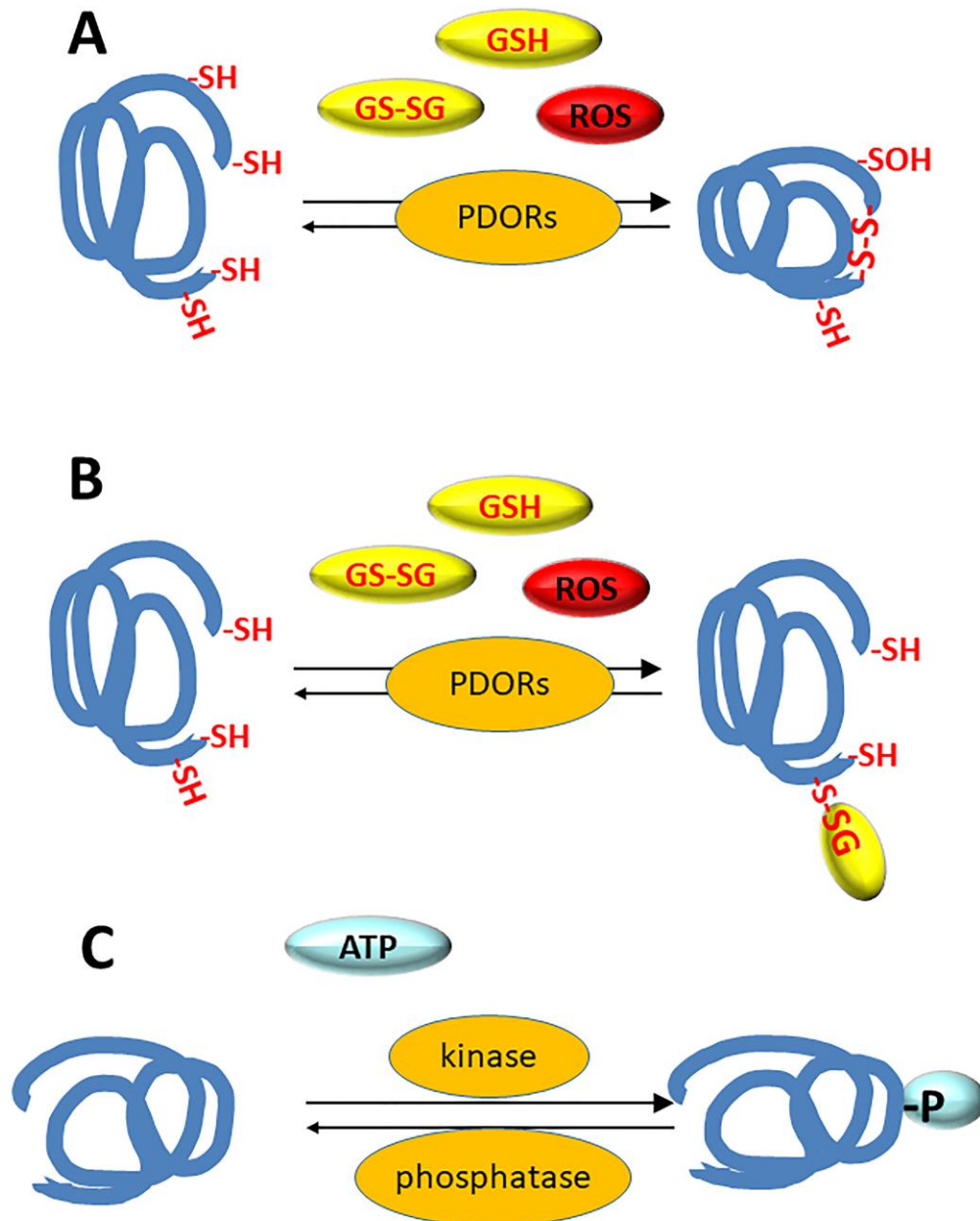


Figure 1. Regulation of protein function by cysteine oxidation. A) Formation of reversible intraprotein disulfides non-enzymatically or by direct oxidation with GSH or thiol-disulfide exchange with GSSG. The panel also depicts the oxidation of a thiol by the action of ROS (the figure depicts the formation of a sulfenic acid, but it could also be a sulfinic ($-\text{SO}_2\text{H}$) or sulfonic ($-\text{SO}_3\text{H}$) acid. B) glutathionylation where mixed disulfides are formed between a cysteine residue in a protein and GSH. C) alteration of protein activity by phosphorylation catalyzed by kinases or phosphatases.

Protein cysteines can also be S-nitrosylated by reactive nitrogen species or oxidized by ROS to form sulfenic, sulfinic or sulfonic acids. Oxidation to sulfinic and sulfenic acid is reversible [20] and may thus have regulatory roles. Enzymes of the family of the protein thiol-disulfide

oxidoreductase (PDOR), such as thioredoxin (Trx), glutaredoxin (Grx), Quiescin-sulfhydryl oxidase (Qsox), or sulfiredoxin (Srx) can catalyze the oxidoreduction of protein thiols and disulfides in either direction, depending on the redox environment [21-23]

One of the first examples of a protein involved in inflammation that can be regulated by formation of an intramolecular disulfide is Keap-1 [24], resulting in activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2; as discussed below). Among the first proteins reported to undergo glutathionylation was the p50 subunit of NF- κ B, as well as others of pivotal importance in regulating the inflammatory response. The top half of Table 1 lists some examples of proteins relevant to inflammation that are subject to regulation by different forms of cysteine oxidation.

Table 1. Key redox regulated proteins in immunity

Reversible disulfide formation				
Modification	Target cysteine(s)	Example	Functions affected	Reference
Intra-protein disulfide	C257, C273, C288, C297	Keap1	Liberation and activation of Nrf2	[24]
Inter-protein disulfide	C54, C347	NEMO	Dimerization, IKK binding	[25]
S-glutathionylation	?	p50	DNA binding	[26]
S-glutathionylation	?	STAT3	DNA and Jak2 binding	[27]
S-glutathionylation	C91	MAL	Interaction with Myd88	[28]
Other reversible forms of oxidation				
Modification		Example	Functions affected	Reference
Methionine oxidation	M358	Alpha-1 antitrypsin	Inhibition	[29],[30]
S-nitrosylation	C15, C20	Surfactant protein D	Activation of pro-inflammatory properties	[31]
S-cysteinylation	various	peptides	Affect MHC recognition	[32]

Although this discussion of molecular mechanisms focuses on protein thiol-disulfides and glutathionylation, other forms of protein oxidation have been shown to regulate inflammation, the most important of which are listed in the lower half of Table 1.

Methionine oxidation to methionine sulfoxide has long been known to inactivate alpha-1 proteinase inhibitor (alpha-1-antitrypsin) [29], as well as other proteins relevant in inflammation and immunity such as interferons and chemotactic peptides (reviewed in [33]). This oxidation is reversed by methionine sulfoxide reductase that reactivates the target protein [34].

It should be noted that most cysteines that are susceptible to glutathionylation can also undergo S-nitrosylation or cysteinylation as shown below:

S-nitrosylation: $\text{P-SH} + \text{XNO} \rightarrow \text{PSNO}$

S-cysteinylation: $\text{P-SH} + \text{Cys-SH} \rightarrow \text{P-s-s-Cys}$

There are examples of both of these post-translational oxidative changes in regulating immune function. S-nitrosylation targets many proteins in the immune system [35] such as surfactant proteins [31], innate immune receptors such as NLRP3 (Mishra et al., 2012; Mao et al., 2013) and signaling molecules such as MyD88 (Into et al., 2008) which is important in inflammatory signaling downstream of Toll-like receptors. Cysteinylation can occur during processing of peptide ligands for presentation on MHC molecules thereby affecting antigen processing and presentation to T cells [32].

Another aspect to be taken into account is that not all cysteines in a protein are equally susceptible to oxidative post-translational modifications. This is determined by several factors including the pK value of the cysteine, which is affected by the neighbouring aminoacids as well as its steric accessibility, particularly in the case of glutathionylation which requires reaction with GSH or GSSG (reviewed in [36]).

4. The immuno-regulatory roles of protein thiol-disulfide oxidoreductases

Protein thiol-disulfide oxidoreductases (PDORs) are enzymes that catalyze the oxidoreduction of protein thiols and disulfides as described above. There are a number of PDORs with different and some overlapping functions. Table 2 lists the main mammalian PDORs.

Table 2. Main mammalian protein thiol-disulfide oxidoreductases.

Enzyme	Symbol	Main reaction catalyzed
Glutaredoxin (old name, thioltransferase)	Grx	Mainly a reductant. Reduction of PSSG to PSH. Needs glutathione reductase and NADPH. $\text{PSSG} \rightarrow \text{PSH} + \text{GSH}$
Thioredoxin	Trx, Txn	Mainly a reductant. Generic reduction of protein disulfides. Needs Trx reductase and NADPH. $\text{PSSP} \rightarrow \text{PSH}$
Peroxiredoxin	Prdx	Prdxs are thioredoxin peroxidases, and catalyze the reduction of H_2O_2 to water using reduced Trx as the electron donor. $\text{H}_2\text{O}_2 + \text{Trx}(\text{SH})_2 \rightarrow \text{H}_2\text{O} + \text{Trx}(\text{SS})$
Protein disulfide isomerase	PDI	Mainly an oxidant. Oxidation of protein thiols to form structural disulfides. $\text{PSH} + \text{GSSG} \rightarrow \text{PSS} + \text{GSH}$
Sulfiredoxin	Srx	Reductant. Reduces Prdx oxidized to sulfinic acid using ATP and a thiol as electron donor; also reduces glutathionylated proteins. $\text{PSOH} + \text{XSH} + \text{ATP} \rightarrow \text{PSH} + \text{XSSX} + \text{ADP}$
Quiescin-sulfhydryl oxidase	Qsox	Oxidizes protein thiols to disulfides by concomitantly reducing O_2 to H_2O_2 . $\text{PSH} + \text{O}_2 \rightarrow \text{PSS} + \text{H}_2\text{O}_2$

PSH, protein thiol; PSSP, protein disulfide; PSSG, protein-GSH mixed disulfide

5. Activities of extracellular protein thiol-disulfide oxidoreductases

There are many lines of evidence demonstrating the involvement of intracellular PDORs in the intracellular signaling pathways downstream of activated pattern recognition receptors on innate immune cells. One of the best examples of this are the Toll-like receptors (TLRs) that, upon recognition of PAMPs or DAMPs, initiate complex intracellular signaling cascades that culminate in activation of the transcription factors AP1 and NF- κ B. Once activated, these transcription factors drive the expression of genes coding for cytokines and other pro-

inflammatory proteins. PDORs play important roles at various points in these signaling pathways as shown in Fig. 2 and reviewed in [37, 38] . It is important to note that the effects of the PDORs in these signaling pathways are reliant on their oxidoreductase activity and not simply due to non-enzymatic effects of these proteins. Thus, changes in the redox states of these enzymes can result in dissociation of molecules that, once released from their associations, can participate in downstream signaling to facilitate changes in gene expression. These redox changes can be viewed as another form of post-translational modification that regulates intracellular signaling in a similar manner to protein phosphorylation.

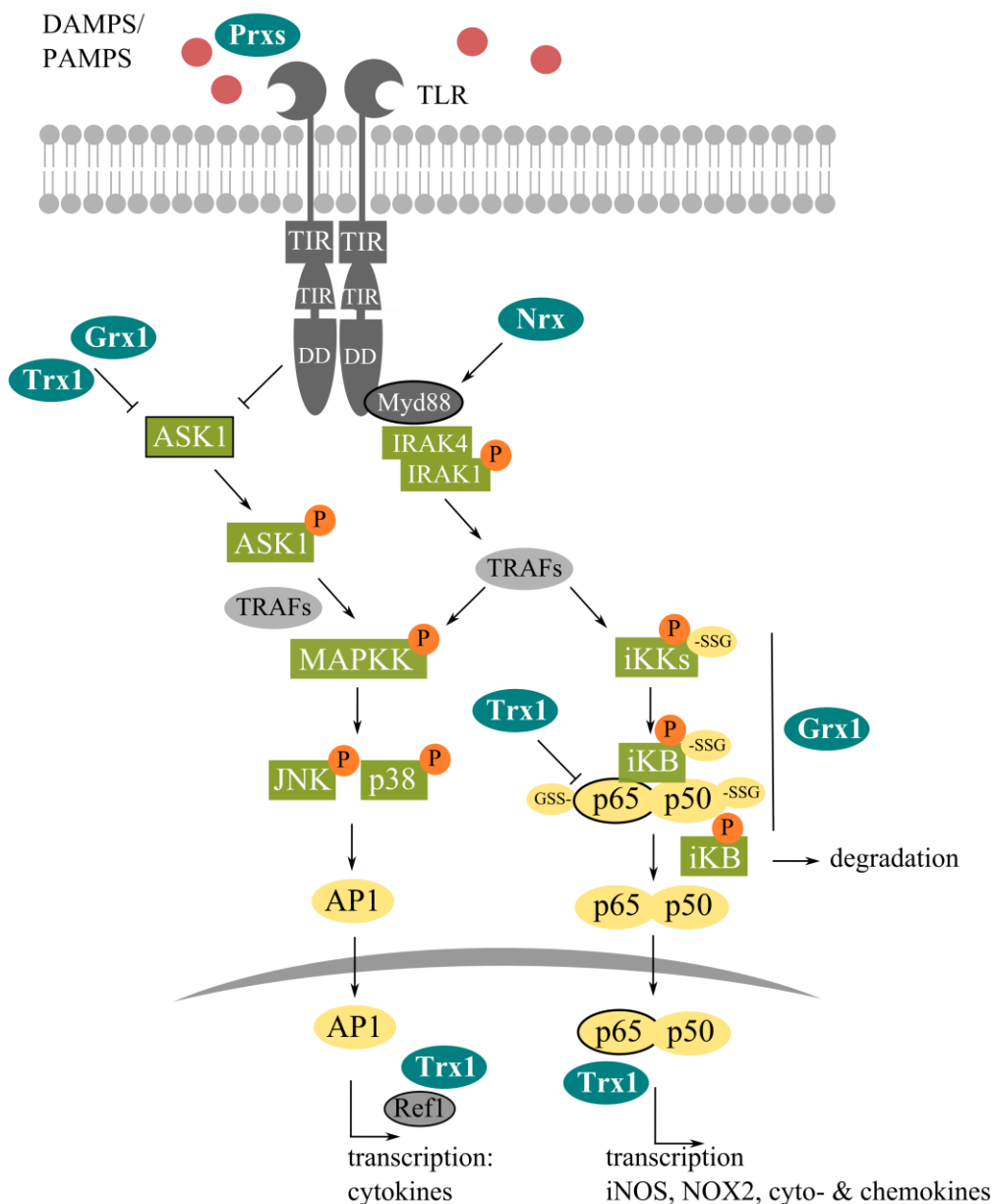


Fig. 2. Regulation of inflammatory signaling pathways by PDORs. Activation of cell-surface located TLRs by pathogen associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) triggers an intracellular signaling pathway that leads to transcription of cytokines, chemokines and other proteins involved in inflammatory responses. Activation of TLRs results in activation of mitogen-activated protein kinase kinase (MAPKK) and dissociation and degradation of inhibitor of kappa B (iKB) resulting in activation of the transcription factors AP-1 (activator protein -1) and NF- κ B (shown here as the p65 and p50 subunits) respectively. The signaling pathway downstream of TLR activation is reliant on sequential phosphorylation of various signaling molecules (shown here by the P in the upper right of the protein name). The points in these signaling pathways that are regulated by PDORs are indicated by the names of the PDORs involved, together with the effect of each enzyme of the activity of the target signaling molecule. Nr1: nucleoredoxin is a Trx-related enzyme. Modified from [37] (CC BY 4.0). Additional abbreviations: activator protein 1 (AP-1); apoptosis signal-regulating kinase 1 (ASK1); c-Jun N-terminal kinase (JNK); damage-associated molecular patterns (DAMP); DD loop (DD); iKB kinase (IKK); interleukin-1 receptor-associated kinase (IRAK); myeloid

differentiation primary response 88 (MYD88); pathogen-associated molecular patterns (PAMP); redox factor-1 (Ref1); Toll-IL-1-receptor domain (TIR); tumor necrosis factor receptor-associated factor (TRAF).

Although PDORs are intracellular enzymes, some of them are secreted under various physiological conditions. However, PDORs may not be enzymatically active in the extracellular environment due to a lack of necessary cofactors (e.g. Trx requires Trx reductase and NADPH; Prdx requires Trx, Trx reductase and NADPH; Grx requires GSH) but have been reported to have cytokine-like activities. In some cases, these activities were described before identifying the hypothetical cytokine as a PDOR. These activities are listed in Table 3.

Table 3. Biological activities of secreted PDORs

Enzyme	Alternative names	Extracellular actions	Reference
Trx	Adult T-cell leukemia-derived factor (ADF)	T-cell proliferation and activation	[39, 40]
Trx		Chemotactic factor (chemokine)	[41] [42]
Trx	Eosinophil cytotoxicity-enhancing factor (ECEP)	Increases antibody-dependent cytotoxicity of eosinophils	[43]
Prdx	(NK) cell enhancing factor (NKEF)	NK cell activation	[44]
Prdx		Induce inflammatory cytokines	[45, 46]
PDI		Thrombus formation; beta 2 integrin regulation	[47] [48]s
QSOX		Regulates extracellular matrix adhesion	[49]

Probably the best such example is represented by Trx. This enzyme was discovered by Arne Holmgren as the intracellular enzyme serving as an essential cofactor of ribonucleotide reductase (reviewed in: [50]), and its sequence was determined in 1968 [51]. Years later, in 1985, the laboratory of virologist Junji Yodoi characterized a cytokine produced by adult T-cell leukemia cells that upregulates the expression of interleukin 2 receptor in T cells, and named it adult T-cell leukemia-derived factor (ADF) [39]. Once the protein was sequenced, four years later, it was found to be identical to Trx [52].

Since then, various other activities of extracellular Trx have been described, most notably as a chemotactic factor [42] and, in its truncated form (Trx80), as a monocyte activator [53, 54]. In addition, extracellular Trx released by antigen-presenting cells generates small-molecular weight thiols via its enzyme action that contribute to T cell activation [55].

Germane to this story, researchers purified a cytokine that stimulated eosinophil antibody-dependent cytotoxic function, and found that the purified protein was identical to Trx [43]. More recently, Prdxs have been reported to be secreted and act as pro-inflammatory agents [45, 56] possibly through activation of Toll-like receptors [57].

6. Redox regulation of signaling molecules

The pioneering studies on NF- κ B mentioned above were the first demonstration of redox regulation of a specific protein in an inflammatory signaling pathway. As indicated in Table 1, several elements of the inflammatory signaling pathway are redox regulated via glutathionylation. Interestingly, earlier ideas of how oxidative stress mediates the induction or maintenance of inflammation, viewed glutathione mainly as an antioxidant and free radical scavenger that would inhibit inflammation (see for instance our earlier studies [12, 58]). However, within the concept of redox regulation glutathione can be seen as a signaling molecule, similar to ATP which is not only an energy-storage molecule but, through protein phosphorylation, is also essential in cellular signaling [59]. We recently described how macrophages depleted of GSH do not show a full response to endotoxin in terms of activation of gene expression profile [60]. If GSH was acting mainly as an inhibitor of ROS-mediated activation of inflammatory pathways, we would expect its depletion to exacerbate the response to endotoxin. However, we found that there were more inflammation-induced genes that require GSH for full induction [60]. While GSH may participate in redox regulation in different ways, protein glutathionylation is one of the most studied mechanisms.

To obtain a list of the genes involved in innate immunity that were reported to undergo glutathionylation, we performed a literature search on August 9, 2019, searching for “glutathionylation or glutathionylated” and each of the 849 in the Gene Ontology term “innate immune response” (GO:0045087) and the 1326 in the Gene Ontology term “inflammatory response” (GO:0006954). The search returned 967 publications and abstracts

were read to identify the proteins reported to undergo glutathionylation. Those not related in any way to inflammation or immunity were excluded. The final list of 41 genes were (by official gene symbol): ALOX5, CASP1, CASP8, CSTA, CYPA, CYPD, DUSP1, ENOS, FAS, GAPDH, IKBKB, IL1B, IL1SR1, ITGA4, JAK1, JAK2, MAL/TIRAP, NCF1, NFkB, NLRP3, NOS3, P47PHOX, PFN1, PON1, PP2A, PRDX1, PRDX2, PTEN, RELA, S100A8, S100A9, SERPINA1, SIRT3, STAT1, STAT3, THOP1, TP53, TRAF3, TRAF6, TTR, TXN1, VIM. For many of these proteins, glutathionylation can affect their activity, as in the case of STAT3 (see Table 1). This indicates that redox regulation may occur at several steps in the immune signalling pathways.

We have analyzed the list of genes above using the reactome analysis tool [61, 62], to highlight specific innate immune signalling pathways susceptible to redox regulation. The results of this analysis are shown in Figure 3, which highlights the signalling pathways involved in the activation of inflammatory signalling where regulation by glutathionylation is involved. Several interleukin signalling pathways (left side of the figure) are overrepresented, as is neutrophil degranulation.

ROS in NLRP3 activation stemmed from early studies demonstrating that inhibition of NADPH oxidase enzymes by diphenyleneiodonium (DPI) reduced IL-1 β secretion in alveolar macrophages or primary human monocytes [65-67]. This was confirmed by knock-down of NADPH oxidase p22^{phox} subunits which resulted in inhibition of IL-1 β secretion in response to monosodium urate (MSU) crystals or asbestos[66]. Together, these data strongly suggested that NADPH oxidase-induced ROS production was critical for activation of NLRP3. However, the necessity of ROS generated by NADPH oxidases for activation of NLRP3 has since been disputed by studies using mononuclear cells from patients with CGD, an immunodeficiency disease caused by defects in phagocyte NADPH oxidase subunits. MSU and silica induced IL-1 β secretion in these cells was unaffected despite the loss of NADPH oxidase induced ROS [68-70]. Mitochondria[71-73] and xanthine oxidase[74, 75] have since emerged as important sources of ROS in activating NLRP3 but there are also several studies that contradict the hypothesis that ROS are necessary for NLRP3 activation and secretion of IL-1 β [76, 77]. Therefore, the exact role of ROS in activating NLRP3 remains a matter of debate, with numerous studies showing conflicting results as summarized in Table 3. It is likely that, like any mechanistic hypothesis, this cannot be generalized and only applies to specific experimental models.

Thus, the current understanding of the role of ROS in NLRP3 activation is unclear and likely involves other strands of redox regulation rather than ROS levels in isolation. A number of elements of the redox regulatory machinery have been shown to be involved in activation of NLRP3 including the antioxidant enzyme SOD1[78], glutathionylation of caspase-1 via increased superoxide levels[79] and activation of Nrf2 resulting in transcription of glutathione, thioredoxin and other antioxidants such as NADPH dehydrogenase 1 and heme[80, 81]. This hypothesis posits that redox modulation, rather than simply ROS levels, is the important factor in IL-1 β processing[82] and provides a possible explanation for the evidence both for and against the involvement of ROS in NLRP3 activation. Furthermore, a wide array of structurally diverse NLRP3 activators have been identified (in addition to MSU crystals), including viral RNA, components of microbial cell walls, nucleic acid, pore-forming toxins, silica, asbestos, ATP and serum amyloid A. The diversity of these molecules make it likely that NLRP3 is not activated by direct interaction with these stimuli, rather by a common cellular event such as dysregulation of cellular homeostasis[83].

Table 4. Evidence for and against a role for ROS in activation of the NLRP3 inflammasome

Cell type	Findings	For / Against	Year, Reference
Alveolar macrophages	Treatment of cells with ATP induces ROS. Inhibition of NADPH-oxidase derived ROS by DPI reduced ATP-induced caspase-1 activation and IL-1 β production	For	2007 [65]
THP-1 cells	Knockdown of p22phox subunit of NADPH-oxidase suppressed IL-1 β release in THP-1 cells in response to asbestos and MSU	For	2008 [66]
THP-1 cells	Extracellular ATP causes NADPH-oxidase induced oxidation and IL-1 β secretion in THP-1 cells. Treatment with NAC and DPI inhibited caspase-1 processing in response to extracellular ATP	For	2004 [67]
THP-1 cells	Inhibition of mitochondrial complex I induced ROS production which correlated closely with NLRP3-dependant IL-1 β secretion. Inhibition of mitochondrial respiration abrogated IL-1 β production and caspase-1 activation	For	2011 [73]
Alveolar macrophages	Inhibition of mitochondrial ROS by SS-31 dose-dependently inhibited cyclic stretch induced caspase-1 activation and IL-1 β production	For	2013 [72]
Murine BMDMs HEK293T cells	Mitochondrial dysfunction is linked to NLRP3 activation and mitochondrial DNA is required for NLRP3 inflammasome-mediated IL-1 β secretion	For	2012 [84]
HEK293T cells THP-1 cells	Redox regulated TXNIP binds directly to NLRP3 in response to MSU exposure. siRNA blocking of TXNIP expression abrogated caspase-1 activation and IL-1 β secretion	For	2010 [85]
Human PBMCs	IL-1 β secretion is ROS-dependant but in an NADPH-oxidase-independent manner. Cells from p22phox- and NOX2-deficient patients had normal IL-1 β secretion	Both	2010 [69]
Human monocytes	IL-1 β secretion is normal in mononuclear cells from patients with chronic granulomatous disease (CGD), a disease characterised by defective NADPH-oxidase enzymes	Against	2010 [68]
Human monocytes	NLRP3 can be activated in a ROS-independent manner in monocytes from patients with CGD. Inhibition of ROS with DPI did not decrease caspase-1 activation or secretion of IL-1 β in cells from these patients	Against	2010 [70]
Murine macrophages	Elevated ROS in SOD1-deficient mouse macrophages inhibited caspase-1 activation. This could be reversed dose-dependently with SOD-mimetics which caused increased ATP-induced IL-1 β secretion	Against	2008 [79]
Murine BMDMs	Nrf2, a redox sensitive transcription factor for a range of antioxidant genes, is essential for inflammasome activation and IL-1 β secretion in response to cholesterol	Against	2011 [80]
Murine BMDMs	IL-1 β secretion in TXNIP-deficient macrophages secreted normal levels of IL-1 β compared to wild-type macrophages in response to MSU, ATP and islet amyloid polypeptide	Against	2010 [86]
Murine macrophages	Inhibition of ROS by DPI and NAC inhibited NLRP3 priming but not activation. Only had an effect when cells were preincubated with ROS inhibitors prior to activation	Against	2011 [87]

Source: [88].

8. Redox mechanisms that affect macrophage polarization in the context of chronic inflammation

Macrophages are extremely plastic cells that play a central role in inflammation and its resolution [89]. The ontogeny, the tissue-specific environment and altered homeostasis at the site of injury, including signals generated by TLR ligands, such as microbial products or danger-associated molecular patterns (DAMPs), are all involved in causing phenotypic and functional changes [90, 91]. Macrophages can be broadly classified into M1 and M2 subtypes [89, 91, 92]. M1 have microbicidal functions and are considered pro-inflammatory whereas M2 promote cell proliferation and contribute to wound healing. M1 respond to TLR agonists and to IFN- γ produced by Th1 cells by activating iNOS and producing NO and ROS to kill pathogens, and secrete pro-inflammatory cytokines, such as IL-8, TNF and IL-1 β , and IL-12 that drives Th1 responses. M2 express high levels of arginase that hydrolyses arginine to ornithine, essential for collagen synthesis and cell proliferation, thus favouring tissue repair and regeneration; they generally produce TGF- β and IL-10 that sustain Th2 responses, and may have anti-inflammatory functions [93-95]. M1 and M2 macrophages are also characterised by different metabolism; M2 rely on mitochondrial oxidative phosphorylation for energy production, whereas M1 are preferentially glycolytic [96-98].

Although M1 and M2 macrophages represent the extremes of a spectrum of different functional subsets described *in vitro* and not necessarily present *in vivo*, it is accepted that an immune response broadly skewed towards M1 (and Th1) polarization may be involved in the pathogenesis of chronic inflammatory disease [89, 99], and greater knowledge of the mechanisms that differentiate M1 from M2 and drive M2 polarization might be exploited for therapeutic purposes.

All the different M1 and M2 functions are driven by a variety of transcription factors. M1 macrophages mainly express STAT1, NF- κ B, IRF5 and AP-1, whereas STAT6, PPAR- γ and IRF4 are predominantly expressed in M2 [91]. HIF-1 is highly expressed in M1 and coordinates glycolytic metabolism and pro-inflammatory cytokine production whereas PPAR- γ in M2 mediates fatty acid oxidation and mitochondrial oxidative phosphorylation [96, 97, 100]. Interestingly, most of these transcription factors (e.g. HIF-1, NF- κ B, STAT6, PPAR- γ) are

redox targets [14, 101-104]. Some specific redox mechanisms that regulate M1 and M2 polarization by affecting pro-inflammatory cytokine production, cell metabolism and/or transcription factor activation are described below.

ROS have been shown to increase pro-inflammatory cytokine production, and therefore M1 polarization, by potentiating LPS-induced MAPK activation [105, 106]; a mechanism might be inhibition of MAPK phosphatases, redox targets that can be inactivated by reversible (and irreversible) oxidation of their catalytic cysteine [107-109]. Also NF- κ B can act as a redox switch to increase M1; in microglia oxidation of the NF- κ B p50 subunit by H₂O₂ decreases its binding to DNA and increases pro-inflammatory cytokine production and M1/M2 balance [102], confirming the predominant inhibitory role of NF- κ B p50 on M1 polarization [110]. In addition, extensive literature has linked ROS to activation of the inflammasome, required for production of mature IL-1 β , highly expressed in M1 macrophages [65-67, 71-73]. However, as discussed above, investigation of the role of ROS on the activation of the inflammasome has produced controversial results (Table 4).

Many redox intracellular targets may be responsible for the impact of ROS on cell metabolism. Mitochondrial oxidative phosphorylation can be inhibited through S-glutathionylation, mediated by Grx2, of complex I of the mitochondrial respiratory chain [111]. In addition, ROS can increase glycolysis by augmenting HIF-1 stabilisation and glucose uptake. In this respect, HIF can be stabilised in normoxia following inactivation of prolyl hydroxylase domain enzymes (PHDs), responsible for targeting HIF- α subunits to the proteasome; inhibition of their activity is due to oxidation of cysteines in the active site [101]. Glucose uptake can be increased by H₂O₂-induced phosphorylation of AMPK and Akt, mediating translocation of the main glucose transporter GLUT4 to the plasma membrane [112]. Inhibition of phosphatases by ROS, also mentioned above, might explain the increased phosphorylation of AMP and Akt [107, 108]. Decreased oxidative phosphorylation and increased glycolysis will result in increased M1 polarization.

All the above evidence points to the possibility that inhibiting ROS might inhibit M1 and induce M2 polarization. However, ROS are also required for induction of M2 polarization, as shown in human and murine macrophages and *in vivo* in mice [103, 113, 114]. Interestingly, in mice with pulmonary fibrosis H₂O₂ produced by SOD1 enhanced M2 polarization by

increasing the transcriptional activity of STAT6, due to redox regulation of a cysteine in its Src homology domain 2 [103].

The fact that ROS may play a role in resolution of inflammation is also supported by the observation that individuals with CGD, characterized by the lack of NOX2-mediated ROS production, in addition to increased infections exhibit also hyperinflammatory responses and an increased frequency of autoimmune disease [115, 116]. Also, polymorphisms affecting Ncf1, the p47 subunit of NOX2, resulting in lower production of ROS, are associated with many autoimmune chronic inflammatory disorders [117], suggesting that ROS might inhibit chronic inflammation. Transgenic rescue of Ncf1 in mononuclear phagocytes of Ncf1-mutant mice inhibited hyperinflammation, demonstrating that ROS produced by NOX2 in macrophages are required for resolution of inflammation [118]. In addition, it is interesting to note that Weyand *et al* reported in a series of studies that T cells from RA patients are ROS-deficient, due to a shift in metabolism; glycolysis is inhibited and glucose is diverted into the pentose phosphate pathway (PPP), resulting in production of lower levels of intracellular ROS and excessive NADPH [119-121]. This “reductive stress” correlates with an inflammatory phenotype and Th1/Th17 polarization, which can be reverted by treatment with oxidants [122].

Although there is evidence that ROS support M1 polarization and a pro-inflammatory response [97, 102, 104, 111], paradoxically ROS-deficiency can result in chronic inflammation. Thus, ROS also seem to favour M2 polarization and resolution of inflammation. A possible mechanism might be through induction of NRF2 [104]. In this regard, recent data have shown that NRF2 activation can enhance M2 polarization [123-126]. It could also be predicted that decreased physiological ROS levels might prevent feedback induction of NRF2, resulting in amplification of the M1 response.

There many studies that describe how changes in the redox extracellular and intracellular environment and redox modifications of transcription factors and signalling molecules can shape the immune response by driving functional changes in macrophages. However, contrasting evidence makes it difficult to reach firm conclusions and to identify redox therapeutic targets [90, 98, 104, 127].

9. Redox regulation in inflammatory disease

The association of inflammation with oxidative stress and vice versa has been recognized for decades and is perhaps unsurprising given the intimate links between aspects of innate immunity, for example the oxidative burst in neutrophils, and ROS levels. However, it has proved difficult to fully elucidate the exact relationship between increases in oxidative stress and inflammation and in particular to determine the extent to which this is a causal relationship. This is an important question because if there is a causal relationship between these conditions, it may be possible to reduce inflammation by alleviating oxidative stress. This may be of particular relevance to diseases where chronic inflammation causes tissue damage and eventual loss of function. There are certainly some diseases where increases in oxidative stress seem to contribute to the pathology, rather than being merely a consequence of increased levels of inflammation. Some of these conditions are discussed below with a focus on conditions where reducing oxidative stress has been demonstrated to ameliorate the symptoms of disease either in appropriate animal models or in humans.

9.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease with a worldwide incidence of about 1%. Although RA is a systemic disease, it primarily affects synovial joints where chronic inflammatory processes can lead to progressive degradation of articular cartilage and eventual bone destruction. The exact causes of RA are unknown, but likely involve a combination of genetic susceptibility and environmental factors that result in a loss of immune tolerance[128]. T cells, B cells and over-production of pro-inflammatory cytokines play key roles in the pathogenesis of RA[129].

Many studies have indicated that increased levels of ROS occur in RA[130], both in synovial fluid [131] and in plasma[132]. However, increases in ROS are only significant if not adequately buffered by antioxidant defenses, in which case the result is oxidative stress and potential oxidative damage to biological molecules and cells. This is indeed the case in RA, where increased oxidative stress is a characteristic of the disease[133-135], and perhaps at least partially explained by the lower levels of antioxidants such as GSH, Vitamin C, thiols[136-138] and the antioxidant enzyme catalase[136] in these patients. Furthermore,

several studies have demonstrated oxidative damage in tissues and synovial fluid in RA, examples of which include lipoperoxidation products[139], oxidative damage of hyaluronic acid[140], oxidation of low-density lipoproteins[141] and protein oxidation[142]. Furthermore, synovial cells from RA patients show upregulation of the transcription factor Nrf2, which is known to be activated in response to oxidative stress and limits cartilage destruction in a mouse model of arthritis [143, 144].

A recent systematic review of the role of oxidative stress in RA concluded that oxidative stress is likely to be implicated in the pathogenesis of RA, and may even serve as a biomarker of disease, but that no firm conclusions could be made due to the heterogeneity of the data [145]. Such heterogeneity of results from a combination of a heterogeneous patient population and the inherent problems in accurately measuring biomarkers of oxidative stress.

An important question is whether oxidative stress and the concomitant oxidative damage is directly involved in initiating and/or maintaining disease. It is possible that the increases in oxidative stress occurring in RA are a result of the inflammatory response that is dysregulated and continually active in disease. However, an interesting finding from a prospective cohort study is that high levels of antioxidants in healthy individuals were protective against the development of RA[146] and at least some studies have reported a correlation between disease activity and oxidative stress in RA patients[147], although in other studies this relationship did not reach significance[148]. More recently, a strong correlation between ROS levels and concentrations of TNF and IL-6 were demonstrated in the serum of RA patients [149]. Therefore, the idea of using antioxidants for therapeutic purposes in RA is attractive.

Several studies have tested the hypothesis that antioxidant supplementation could be used to therapeutic benefit in RA. Most studies have used measurements of MDA and TAC as measures of oxidative stress and concentrations of TNF and/or IL-6 as measures of inflammation. Treatment of RA patients with antioxidants such as NAC[150], Vitamins A and/or E[151, 152] or CoQ10[153] did reduce the levels of MDA in the blood and in some, but not all, cases the levels of TAC. However, in those studies that measured disease activity using the DAS28 score, there was no significant change indicating that there was little effect of the antioxidant supplementation on clinical disease.

Animal models of RA have been used to good effect to elucidate the relationship between oxidative stress and the disease process. Arthritis in these models is certainly associated with increased oxidative stress, mirroring the human disease, with increases in iNOS and MDA and decreased Gpx and SOD activity in the affected joints [154]. Intriguingly, local overexpression of antioxidant enzymes (SOD3 or catalase) ameliorated arthritis in rats with antigen-induced arthritis[155] or mice with collagen-induced arthritis[156] suggesting that oxidative stress is directly involved in the pathogenesis of disease. The mechanisms involved are less easy to understand. The pro-inflammatory cytokines TNF and IL-6 are key mediators of inflammation in RA, but the concentrations of the cytokines were not affected by the increased SOD3 or catalase. The protective effects of the antioxidant enzymes were instead due to decreases in catabolic enzymes such as matrix metalloproteinases that are up-regulated in joints affected by arthritis. The decrease in clinical score was thus a result of decreased matrix and cartilage degradation rather than inhibiting inflammation[155]. It is also important to note that the most successful outcomes on disease activity in rodents were achieved by local overexpression of antioxidant enzymes via gene transfer, an approach not yet possible in humans. Local reduction of ROS and oxidative stress in the effected joints may be necessary for therapeutic effects in RA which would explain why the clinical trials described above where systemic administration of antioxidants had little effect on disease activity.

9.2. Gout

Gout is an inflammatory arthritis driven by the production of the pro-inflammatory cytokine, IL-1 β . It has a worldwide prevalence of 0.1-10 %[157] and is characterized by episodes of acute inflammation generated in response to monosodium urate (MSU) deposits in synovial joints. Uric acid is the major antioxidant in the blood, contributing around 60% of the free radical scavenging capacity[158]. During periods of hyperuricemia, when the concentration of serum uric acid increases, crystals of uric acid form and are deposited in synovial joints. It is these crystals that are recognized by innate immune cells and result in activation of the NLRP3 inflammasome that processes pro-IL-1 β for secretion. The inflammation associated with an acute gout attack is driven primarily by secreted IL-1 β [159].

Interestingly, although the greatest risk factor for gout is hyperuricemia, itself an antioxidant, gout is associated with increased oxidative stress as indicated by higher MDA levels and lower SOD and catalase activities compared with healthy controls[160]. At a cellular level, MSU crystals induce the production of ROS in human synoviocytes [161] and monocytes [88]. This ability of MSU crystals to induce ROS production is significant as ROS have been postulated to be involved in activation of NLRP3 which is necessary for secretion of IL-1 β . However, as discussed above, this relationship between ROS levels and activation of NLRP3 is still unclear.

The front-line treatment for gout attacks is allopurinol which inhibits the activity of the enzyme xanthine oxidase thereby preventing the production of uric acid from xanthine. Interestingly, allopurinol treatment also decreases oxidative stress in gout patients, possibly by reducing the production of ROS from the xanthine oxidase reaction[160]. However, it is difficult to fully elucidate the pathological importance of oxidative stress in gout. Allopurinol is therapeutic presumably by lowering uric acid levels and possibly by decreasing oxidative stress, but it is difficult to ascertain the relative contributions of these actions to the therapeutic benefit.

9.3. Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that affects approximately 2.5 million people worldwide. Disease is characterised by relapsing-remitting episodes following peripheral immune attack on the CNS. This causes inflammation in the grey and white matter of the CNS leading to destruction of myelin in the brain and spinal cord. Demyelination and persistent glial cell-mediated neuroinflammation within the CNS contributes to the accumulation of progressive neurodegeneration and disability[162].

A large and convincing body of literature exists on the importance of oxidative stress in MS pathophysiology. MS is particularly interesting in this regard because oxidative stress appears to be a fundamental and possibly causative element of disease as opposed to being a by-product of inflammation as may be the case in other diseases. There are multiple accounts of increased oxidative stress in MS patients, both in the CNS [163-166] and the periphery [167-

170]. Markers of oxidative damage in MS brain tissue include oxidative damage to cellular membrane lipids[166], generation of peroxynitrite which damages proteins and oxidative damage to nucleic acids by the formation of 8-hydroxy-2'-deoxyguanosine[171]. Furthermore, chronically-activated microglial cells produce reactive oxygen species (ROS) that can influence mitochondrial function and energy metabolism that can then result in autoimmune-independent neurodegeneration [172].

It is interesting that a number of antioxidant enzymes such as superoxide dismutase (SOD), catalase, haem-oxygenase [166] and peroxiredoxins 2[173] and 6[174] are upregulated in MS, probably as a result of chronic oxidative stress. Peroxiredoxin 6 not only protected against inflammation in a mouse model of MS, but also against disruption of the blood brain barrier by inhibiting the activity of MMP9[174], similar to the ability of catalase and SOD3 to reduce cartilage degradation in a mouse model of RA by inhibiting MMPs[155]. Indeed, antioxidant enzymes have been tested in animal models of experimental optic neuritis [175-177] or MS i.e. experimental autoimmune encephalomyelitis (EAE)[174, 178] for therapeutic effects and have been shown to ameliorate the symptoms of disease. Unsurprisingly, a combination of antioxidant enzymes (e.g. SOD and catalase) was more effective in reducing the clinical signs of EAE than either enzyme alone [177]. Notably, all of these effects of antioxidant enzymes that have been studied *in vivo* have relied on the generation of transgenic mice or overexpression of enzymes via gene therapy with adenoviral vectors or engineered syngeneic cells[155]. Although these approaches are very useful for proof-of-concept testing in animal models, they are not yet appropriate for treating human disease.

However, the importance of oxidative stress in MS and the potential therapeutic benefits of reducing this is underlined by the fact that one of the front-line treatments for MS, dimethyl fumarate (DMF) exerts therapeutic effects at least partially by reducing oxidative stress via activation of Nrf2[179, 180]. Furthermore, dietary intake of exogenous antioxidants such as flavonoids and α -lipoic acid reduce the clinical signs of disease in animal models of MS[181, 182] and there is now substantial interest in the use of oral antioxidants for improving the clinical symptoms of MS [183].

Conclusions

The studies summarized in this review clearly indicate that redox modifications are involved in the regulation of innate immunity and inflammation. This highlights the role of endogenous ROS and redox reactions in regulating immune processes, indicating that use of broad-spectrum ROS scavengers may affect important signaling pathways. It should be mentioned that, so far, no specific antioxidant molecule has been approved for clinical use in any of the inflammatory diseases mentioned in this review, and there were no relevant Cochrane reviews available at the time of writing. On the other hand, two Cochrane reviews on the use of antioxidants in two acute inflammatory conditions (acute respiratory distress syndrome and systemic inflammatory response syndrome) did not recommend their therapeutic use [184, 185]. Even the assumption that the pharmacological action of DMF in MS is mediated by activation of Nrf2 has been challenged by a study showing its effectiveness in a model of MS in Nrf2-knockout mice [186].

Nonetheless, there is strong evidence that oxidative stress may in fact occur in several inflammatory diseases and it is possible that it may represent a druggable target for at least some of them. However, in view of the literature discussed in this review, it is probable that decimating ROS levels with antioxidant scavengers may result in several non-specific effects, and current studies point to the necessity for more targeted strategies such as specific inhibition of ROS-producing enzymes involved in disease that would leave the other ROS-generating pathways intact [187, 188].

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